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DOI: https://doi.org/10.2174/157016112798829832

Current Vascular Pharmacology, 2012, 10, 107-124

"Endothelial Progenitor Cells" as a Therapeutic Strategy in Cardiovascular Disease

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Cardiovascular atherosclerotic and ischemic diseases constitute the leading cause of morbidity and mortality throughout middle- and high-income countries. More efficient preventive and regenerative therapeutic strategies are therefore urgently needed. The repeated finding that putative "endothelial progenitor cells" (EPCs) can efficiently promote angiogenesis and restore perfusion of ischemic tissues has provoked a wealth of studies evaluating and developing their therapeutic potential. In the present review, we discuss the growing knowledge about various distinct cell populations which have been collectively termed "EPCs", including myeloid cells and progenitor cells of different origin. We also present clinical studies aiming to examine their therapeutic potential for cardiovascular disease. In addition, we will discuss recent insights into mechanisms leading to dysfunction of "EPCs" in cardiovascular disease. Those findings may help to optimize autologous cell-based treatment approaches, as well as to establish cellular dysfunction itself as an interesting novel therapeutic target.

Keywords: Cell therapy, cardiovascular disease, cell dysfunction, endothelial progenitor cells.

1. INTRODUCTION

Impaired endothelial cell integrity and function play a major role in atherosclerosis, as well as in ischemic cardiac and peripheral disease. The need to maintain endothelial function and integrity throughout life is challenged by the constant exposure of the endothelium to recurring noxious influences, such as cigarette smoking, dyslipidemia and hyperglycemia that induce increased endothelial reactive oxygen species (ROS) generation ([1, 2], reviewed in [3]).

Cardiovascular pathologies may result from deterioration of endothelial function and integrity in both, the microcirculation as well as the macrocirculation. As microvascular endothelial cells (EC) become dysfunctional and apoptotic e.g. in patients with diabetes mellitus (DM) or hypertension, capillaries in vital organs as well as in limbs dissociate, which results in inadaequate tissue perfusion and impaired wound healing [4, 5]. In the macrovasculature, endothelial dysfunction i.e. impaired endothelial nitric oxide (NO) availability, is a hallmark of early atherosclerosis. Today's conception is that constitutive or recurring endothelial activation, in particular in regions of oscillatory shear stress or turbulent blood flow, is characterized by upregulation of adhesion molecules, loosening of EC-EC junctional bonds, increased generation of ROS and impairment of NO availability, which finally induce and maintain an increased inflammatory state of the vascular wall. As a result, the enhanced recruitment of leukocytes and inflow of plasma lipids and lipoproteins into the sub-endothelial space promote the formation of atherosclerotic plaques [6]. Progressing plaque growth, together with plaque rupture or erosion and thrombosis induce acute vessel occlusion, such as in myocardial infarction (MI) or ischemic stroke. While acute treatment focuses on the restoration of tissue perfusion e.g. by thrombolysis or percutaneous intervention, the induction of reparative angiogenesis i.e. the growth of new microvessels into the tissue at need, is considered a promising strategy to confine post-infarction cardiomyocyte (CM) loss. This strategy crucially relies on the endothelium as a main player.

Although today we have several pharmacological tools available to combat the progression of CVD and to enhance survival following acute cardiovascular events, a true regression of atherosclerosis as well as cardiac repair after MI or in ischemic heart disease are not sufficiently achieved by currently established treatments. Therefore, different cell-based therapies i.e. transplantation of immature cell types, such as stem and progenitor cells (S/PC) have been investigated for ischemic organ or tissue repair with great enthusiasm [7]. While the initial high hopes placed in this treatment have not yet been clearly fulfilled, we have gained first clinical experience, mostly with autologous bone marrow cells (BMC), including the establishment of safety of the procedure and the achievement of a significant, though rather small improvement of cardiac function after an acute MI. We have also achieved a better understanding of the cellular and molecular mechanisms underlying the action of various cell populations, including the so-called "endothelial progenitor cells" (EPC). As described below, this term has been used for several different cell populations, and not all of these are "true" endothelial progenitors (in the sense that they differentiate into mature endothelial cells in vivo), but may rather promote endothelial growth in a paracrine fashion. The field is now advancing towards an optimization of treatment protocols, which are better adjusted to the individual pathology and characteristics of the donor/recipient, and is focusing on overcoming important limitations of cell-based therapies, including ways to establish ex vivo technologies to improve the functional capacity of patient-derived autologous cells.

2. WHAT ARE "ENDOTHELIAL PROGENITOR CELLS"?

In the early 1990s, endothelial cell progenitors were largely examined in embryonic development of the cardiovascular system and the term was applied to cells with a low degree of differentiation, but a high potential to differentiate towards endothelial cells. These cells were also termed angioblasts [8]. In 1997, Asahara and colleagues reported the existence of circulating cells with endothelial lineage potential - i.e. putative "endothelial progenitor cells" – in the postnatal setting i.e. within enriched CD34+ cell fractions obtained from the peripheral blood of human donors [9]. Despite the in vitro endothelial differentiation potential of postnatal CD34+ cells, only few of these cells or their progeny were detected at sites of neovascularisation after transplantation in in vivo ischemia models. In comparison, the observed functional effects (considerably increased neovascularization and blood flow) were beyond what could be expected from differentiated progenitor cells replacing lost EC, or generating new ones [10]. Further findings underlined the importance of paracrine effects of the putative "EPCs" for the observed functional improvement i.e. reduction of ischemia [11-13].

Methodological and terminological inconsistencies have contributed to the fact, that severeal different cell populations have been termed "endothelial progenitor cells": After the late nineties, so-called "EPCs" were obtained by subjecting unsorted peripheral blood mononuclear cells (PBMC) to adherence culture in endothelial growth medium immediately, omitting the CD34+ enrichment step [14]. The resulting cells had both, monocyte markers such as CD14, and endothelial markers such as VE-cadherin [14]. Later work verified the myeloid identity of the outgrowing cells [11, 15]. However, the majority of the community adopted the term "endothelial progenitor cells" for these PBMC-derived cells and also discussed their possibilities in this context, fuelled by the cells´ therapeutic capabilities which was by now beginning to be verified in human patients [14, 16, 17].

Minute characterization of PBMC-derived cells has established that two - still heterogeneous – populations of cells with angio-supportive characteristics can be obtained from adult peripheral blood: "early EPCs" or "early outgrowth cells" (EOC), which adhere to fibronectin-coated plastics within 4-7 days, at which time colony-like cell clusters can be observed; and "late EPCs", also termed "endothelial colony-

forming cells (ECFC)", which adhere to collagencoated plastics faster (within two days), but take longer (two to three weeks) to form proliferating colonies [13]. EOC (or "early EPCs") mainly consist of monocytic cells, which do not give rise to mature EC, but integrate into the subendothelial space of an injured host vessel wall after transplantation - a feature that reminds of resident "patrolling" monocyte subtypes [18, 19]. In contrast, ECFC (or "late" EPCs) contain only minimal amounts of myeloid cells, while a larger protion of them can differentiate into cells of endothelial phenotype [12, 13, 20, 21]. Similar functional characteristics seem to apply to CD34+ vs. CD14+ circulating cell types, freshly isolated from the circulating blood [22]. Of note, the role of monocytes indeed seems to be fundamental to the architecture of newly generated vascular networks, due to their ability to form linear and branched structures in three-dimensional matrix and to interact with various cell types of vascular and non-vascular identities, thus guiding the course of the neovessel [23].

Apart from circulating cells, derived from the bone marrow (BM) or other organ stem cell niches, various types of vascular resident S/PC have been identified. They may contribute to angiogenesis,

population of vascular grafts by recipient cells, and possibly also to vessel wall remodelling and atherosclerosis [24-28]. Their role in physiology and pathophysiology seems to be underestimated as far as now, especially in the context of stent healing or in-stent restenosis [29].

For this review, the term “endothelial progenitor cell” will be applied as a generic term including “true” endothelial progenitors - circulating and resident - which are able to give rise to endothelial cells as well as to myeloid EOC, that rather act in a paracrine fashion on the endothelium. For clinical studies, we will largely refer to experience with autologous BMC, although they clearly constitute a heterogeneous cell population containing mature cells in addition to mesenchymal and hematopoietic progenitors (MSC, HSC, respectively).

This concept reflects the two directions in which research on “EPCs” progresses: (1) the ongoing search for the "true" angioblast/endothelial progenitor cell, driven by the motivation to understand underlying cellular processes of vascular pathologies and regeneration, as well as to identify a defining set of antigenic markers [30], and (2) the clinically motivated search for the therapeutically most efficient cell(s), not necessarily willing to await improved mechanistic understanding, but with the chance to speed up the development of potentially efficient therapies. Yet, mechanistic understanding, including cellular identity, is critical to clinical application, in order to improve efficiency and safety, while economic, ethical and logistic aspects might hinder the transgression of specific cell types into the clinical setting, which are nevertheless highly suited in in vitro and pre-clinical studies, such as embryonic SCs.

3. THERAPEUTIC POTENTIAL AND NON-TARGET EFFECTS OF “ENDOTHELIAL PROGENITOR CELLS”

The contribution of different types of endothelial supportive cells to tissue healing and/or regeneration varies between individual pathological settings. Maintenance or restoration of a confluent, non-activated endothelial layer requires long-acting mechanisms considering also the delicate interactions between various cell types. Monocytes/EOC via their capacity to phagocytose dysfunctional or dying cells, to export cholesterol from the vessel wall, and to secrete distinct spectra of cytokines, play an important role here [11-13, 31, 32]. In contrast, regeneration of the postinfarction myocardium, again crucially involving the phagocytotic and paracrine activity of monocytes, progresses in two distinct stages: first, a fast “clean-up” of dead and dying cells, and later on, the stimulation of repair i.e. angiogenesis and thereby enhancement of tissue perfusion [33, 34]. Other EOC- and BMC-mediated mechanisms include remodelling of the extracellular matrix and possibly the enhancement of cardiomyocyte (CM) survival and functionality by paracrine mechanisms [35-39].

The qualitative and quantitative composition of the “EPC”- or BMC-derived secretome crucially affects short and long-term therapy success. In general, cytokines secreted by the recruited leukocytes are considered to beneficially impact on infarct size and cardiac function after MI [40]. On the other hand, leukocyte-derived cytokines have been associated with post-MI microembolisation and may affect vessel maturation [41-47]. Following MI, the time interval between the acute event and cell application is characterized by alterations in milieu conditions i.e. a high inflammatory load within the first week

followed by a more repair-promoting milieu at later stages [33, 34]. BMC transplantation after the first week post-MI could therefore be more effective [48], a concept which is currently examined prospectively in clinical trials in patients after MI (SWISS-AMI, TIME).

As “EPCs” may support vascularisation of an ischemic tissue, so they may do with a (silent) tumour, potentially facilitating tumour growth [49, 50]. In this context, the risk of de novo tumour formation/leukaemia induction, which has been observed after transplantation of foetal stem cells or cord blood cells [51, 52], might be reduced by using committed progenitor cells, or differentiated (autologous) adultderived cells [53, 54]. Although angiogenesis in the ischemic heart or peripheral limbs might be a therapy target of BMC/”EPC” transplantation, angiogenesis at developing or mature plaques is unwanted, as it may confer plaque instability [55-57]. Even in the absence of noticeable angiogenesis, recruited HSC/myeloid EOC may facilitate intima thickening, destabilize plaques or promote calcification [58-61]. Likewise, circulating or resident progenitor cells may contribute to intima thickening [62]. Although, in the current clinical trials of BMCs in patients after MI, no safety concerns have been raised, this aspect always needs to be taken into account for cell-based therapies.

Immunological aspects hinder the usage of allogenic donor cells, while embryonic stem cells -although harbouring favourably high differentiation potential and low immunogenic risk - are potentially tumourigenic and raise ethical concerns, further aggravated by the opposition of high numbers of potential recipients and the low number of in-hospital abortions. From ethical, economical and safety aspects, autologous cell sources therefore remain as the most practicable cellular source. Indeed, cells with angiogenic/vascular potential have been detected even in CVD patients, albeit cellular functionality negatively correlates with cardiovascular risk, leaving the least potent cells to the patients who would need the most functional ones [63-65]. Two strategies can be derived from those considerations: the protection of stem cell niches throughout life needs to be addressed prospectively [66]. For the individual patient in need for treatment, ex vivo “priming” or engineering of autologous cells might provide more efficient approaches in the near future. Various strategies have been devised towards that end, including improvement of isolation and storage protocols for the selection of the most efficient cell type and their and functional preservation [67, 68] as well as ex vivo pharmaceutical pre-treatment or genetic engineering of cells prior to re-implantation. Improving the number of cells to reach the target tissue and to persist there e.g. by optimizing the route of transfusion or accompanying pharmacological treatment (e.g. CD26 inhibitors), might further improve efficiency. Below, we will discuss distinct strategies to employ endothelial growth promoting cells in distinct pathological situations.

3.1. “EPCs” to Treat Microvascular Dysfunction and Rarefaction

Microvascular rarefaction i.e. the reduction and loss of a functional network of capillaries normally ensuring organ perfusion, likely contributes to different forms of cardiomyopathy, including ischemic or hypertensive cardiomyopathy and diabetic cardiomyopathy. Deteriorating microvasculature looses pericyte coverage and assumes a tortuous phenotype with discontinuous endothelium (Fig. 1) [69]. Although at the first glance paradoxical, the loss of capillaries in heart, kidney and peripheral tissues shares some molecular and cellular mechanisms with diabetic retinopathy, such as increased capillary permeability and tortuosity (reviewed in [70]).

The demonstration of the pro-angiogenic potential of “EPCs” has therefore raised hopes for a novel treatment strategy, especially in those patients who do not respond to other modes of therapy [16]. However, cellular functions of “EPCs” are severely affected i.e. in diabetic patients and smokers, thus potentially limiting the efficiency of autologous cell transplantation [64, 65]. Ex vivo modification of cellular function prior to re-introduction (Table 1) might therefore harbour specific benefits for patients with an impaired “EPC” function. As an example, the overexpression of platelet-derived growth factor (PDGF), a mediator of pericyte-EC interaction during microvessel stabilization, has been proposed. Interestingly, ex vivo PDGF treatment of human CD34+ progenitor cells enhanced their homing in a diabetic mouse model and promoted in vitro the emergence of adherent cells/clusters that expressed the endothelial markers VE-cadherin and CD31 [71]. Ex vivo overexpression of growth factors (e.g. vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF-1)) [72, 73], oxidative defence effectors (e.g. manganese superoxide dismutase), endothelial nitric oxide synthase (eNOS), or PPAR.. activators served to improve cellular functions in in vitro studies and animal models [72-76]. Transfer of these experimental approaches into the clinical setting might come closer with the development of safer vectors and the availability of first results from clinical trials employing direct and cell-mediated gene therapy (reviewed in [77, 78]). Alternatively, biodegradable matrices could be adopted as a means for time and dosage-dependent release of mature proteins e.g. for topical application in diabetic wound healing [79]. Furthermore, systemic effects are exerted by lifestyle approaches, such as physical exercise training or flavonoids in certain foods (tea, red wine, chocolate), as well as drugs with pleiotropic actions. Especially statins have been shown to be effective in improving “EPC” availability and function via activation of the PI3K/Akt/eNOS pathway, besides potent anti-inflammatory effects and blockade of the ratelimiting enzyme of cholesterol synthesis, HMG-CoAreductase [80-82].

The kallikrein-kinin-system has gained attention for its role in regulating angiogenesis, as well as vascular stabilization [83-85]. Cellular players are mature cells of the vasculature, such as EC or SMC, but also EOC, as well as individual progenitor and leukocyte subtypes, which regulate angiogenesis in a paracrine manner. In a non-diabetic mouse model, expression of the kinin B2 receptor was necessary for the recruitment of progenitor cells to ischemic tissue and initiation of capillary growth and reperfusion [86]. In vitro, EOCs migrated towards the B2 receptor ligand bradykinin (BK) and were enriched out of the heterogeneous PBMC by using BK as a chemoattractant. Progenitor cells from patients with CVD could not be enriched by BK-induced migration [86]. Interestingly, progenitor cells and monocytes from young type 1 diabetic patients without CVD retained B2 receptor expression and BK-induced migration, while individual B2 receptor-mediated cellular functions, such as NO generation, were impaired [87]. Uncoupling of the B2 receptor from downstream signaling was associated with reduced expression of tissue kallikrein, a protease mediating kinin generation and matrix degradation, leading to impaired invasive capacity of EOCs from type 2 diabetic patients [88]. Consequently, ex vivo overexpression of both, kallikrein and the B2 receptor, in diabetic EOC did restore invasive capacity [88]. While interactions between the members of the kallikrein-kinin system may be complex and simple overexpression of one component might not suffice to restore angiogenesis in certain pathologies [88, 89], combinatory treatment, together with re-coupling of eNOS, might enhance EOC pro-angiogenic capacity.

Maturation of the nascent vascular network i.e. the recruitment of pericytes and SMC to provide stability and reduce permeability is crucial to ensure functionality of newly generated vasculature [90]. To achieve both, initiation of angiogenesis and subsequent pericyte recruitment, VEGF and PDGF have been successfully applied as dual gene therapy in various animal models [91, 92]. Recent work furthermore indicates an additional role of the kinin B2 receptor in vessel maturation by pericyte/SMC recruitment [84]. The intrinsic capacity of “EPCs”/EOCs to counteract capillary permeability by stabilizing tight junctions, as was shown recently for myoeloid multipotent progenitor cells, might be enhanced by tailored modification of autologous “EPCs”' paracrine activity prior to reintroduction e.g. by cotransfection with different growth factor genes [93].

Besides 'classical' paracrine substances, further mediators of endothelial function, supplied by “EPCs”, as well as alternative routes of delivery have been identified. Interesting novel data have indicated that actively shed membranous vesicles, depending on their size classified into microparticles (MP), exosomes, or microvesicles, may carry soluble proteins, membrane proteins, lipids and nucleic acids, thus mediating communication between the cell of origin and the target cell over variable distances [94-98]. Exogenously modified “EPC”, which shed MPs, or the “EPC”-derived MPs themselves are therefore considered promising tools for targeted drug delivery [99]. This principle could be extended to ex vivo engineering of “mother cells” shedding MPs containing a desired protein or microRNA profile. The cells would then be available for producing multiple doses of paracrinally active “conditioned medium” to be transplanted during several treatment sessions, which might increase efficiency of the therapy [100].

Therefore, by their intrinsic paracrine activity, “healthy” EOCs might counteract the destabilization of microvascular networks, as well as support the re-constitution of a mature, stable network of new capillaries (Fig. 1). Ex vivo engineering or pre-conditioning of patients autologous EOCs (i.e. by overexpression of pro-survival factors and/or chemoattractants, or exposure to survival-promoting drugs (Table 1)) prior to transplantation might be helpful, not only to promote angiogenesis, but also to enhance stability of newly generated or pre-existing micro-vessels.

3.2. Potential of “EPCs” as a Therapeutic Strategy for Macrovascular Disease

As for the establishment of a functional neovasculature, the interplay between endothelial and sub-endothelial cells of the vascular wall is critical for the maintenance of macrovascular function. During life, various factors, such as circulating lipids and glucose, turbulent flow due to vascular geometry, and inflammatory cells activated by environmental toxins, add up to establish permanently inflamed areas of the vascular wall, predominantly at bifurcations. This is followed by intima thickening due to the immigration of leukocytes from the blood and SMC from deeper layers of the vessel wall. Inflammation is further promoted by leukocytes entering the plaque via vasa vasorum. The loss of endothelial function and integrity plays a crucial role in atherogenesis: Endothelial-derived NO generation, important for controlling SMC relaxation and proliferation, is reduced and the tight coupling of endothelial cells through junction molecules, as well as low expression of adhesion molecules, are reverted. In consequence, plasma proteins can leak into the subendothelial space, followed by leukocytes which invade the vessel wall. Therefore, the reconstitution of endothelial confluence and quiescence is regarded key in prevention and halting of atherosclerosis, while its reversal might be achieved by enhancing reverse cholesterol transport e.g. by HDL [101]. Towards this end, functionality

and survival of EOC when exposed to the pro-apoptotic conditions within the inflamed vessel wall are critical for effectively counteracting atherosclerosis [102].

The role of “EPCs” in counteracting, but also potentially in promoting atherosclerosis seems to be conveyed mainly by their paracrine action Fig. (2). They can attenuate vascular inflammation e.g. by interleukin-10 secretion, support endothelial cell turnover e.g. via NO generation or secretion of interleukin-8, hepatocyte growth factor or VEGF [11], or by promoting reverse cholesterol transport [103]. Special attention has been given to the capacity of EOCs for NO generation, which is considered a hallmark of their functional capacity [19, 104-106]. Indeed, “EPC” may loose the capacity for NO generation well before the onset of vascular disease in persons with increased risk, such as diabetes mellitus or pre-hypertension [19, 87]. Paracrine dysfunction of “EPC” in persons with enhanced cardiovascular risk (e.g. in smokers or diabetic patients) therefore may impair endothelial functions two-fold [107-109]. In the chronic setting, treatment options such as statins or exercise training, which can improve “EPC” paracrine actions in vivo without need of cell extraction, ex vivo modification and re-implantation, seem favourable, as drugs/lifestyle modifications can be applied over long time courses. In contrast, single cell transplantation treatments may only cover shorter time periods. However, cell-derived non-immunogenic products, such as microvesicles loaded with specific proteins or microRNA, might be suitable for repeated administration. Detailed investigation of microvesicle composition and the systemic effects exerted by individual microRNAs in CVD are therefore awaited with great expectations.

As discussed elsewhere [110], percutaneous coronary intervention (PCI) induces severe damage to the endothelium i.e. endothelial denudation and stretching of matrix and underlying cell layers. The fast re-establishment of a confluent, quiescent endothelial layer is therefore accounted crucial for both, the attenuation of atherosclerosis, as well as the prevention of in-stent re-stenosis/thrombosis. Antibodymediated capturing of circulating CD34+ progenitor cells by implanted stents is currently followed as a potential strategy to promote endothelial healing after stent implantation [111, Current Vascular Pharmacology, 2012, Vol. 10, No. 1 113

112]. Although safety and feasibility were suggested by several studies [113-115], higher rates of target vessel failure in patients receiving the “EPC-capture-only” stent were described for high risk lesions as compared to drug-eluting stents [116-121]. Therefore, a new generation of combined CD34+ attracting and drug-eluting stents is currently entering clinical trials (REMEDY, REMEDY-OCT) with the hope to achieve accelerated endothelial healing without increasing restenosis risk [122].

While progenitor cells circulate in the blood only in low numbers, resident S/PC within adjacent vascular sections provide a more readily available source for re-population of vascular grafts, and possibly also for the reendothelialisation of stents or - with adverse result - intima growth [24, 25, 27, 123, 124, 28]. Vascular resident S/PCs are positioned where they can rapidly provide support to damaged endothelium, but naturally they will be equally affected by blood-borne stressors, like inflammatory mediators increasing oxidative stress within the vessel wall, and may therefore suffer equally [125]. Consequently, bone marrow-derived progenitor cells, leaving their protective environment and entering the circulation, as well as vascularresident progenitor cells, will both be exposed to different factors, leading to cellular dysfunction [126].

Statins achieve reduction of cholesterol levels by blocking HMG-CoA reductase, but they also activate the prosurvival Akt/eNOS pathway and skew EOC differentiation towards an repair-promoting phenotype [81, 127]. In the setting of stent implantation, concomitant statin therapy serves to accelerate re-endothelialisation and reduce intima thickening and thrombosis, improving survival after PCI [128-131]. Contributing to those effects might be the increased mobilization, as well as better homing characteristics of EOC and progenitor cells (Table 2) [127, 132, 133].

Similarly, physical exercise training improves endothelial function in various physiological and pathological settings [134-136]. Exercise-induced effects are mainly ascribed to a systemic decrease of inflammatory agents, increased NO availability, downregulation of NADPH oxidase and upregulation of enzymes involved in antioxidative defence, but recent studies have also established higher numbers of circulating “EPC” [137-145]. Aortic valve stenosis represents another major cardiovascular pathology, which shares underlying mechanisms with atherosclerosis, such as leukocyte infiltration and endothelial deterioration, but calcification processes play a larger role than in atherosclerosis [146, 147]. The progression of valve disease, characterized by EC loss, coincides with reduced survival and lower circulating numbers of “EPC” [148]. In this context, exercise training served to prevent aortic valve disease in a high-cholesterol induced mouse model of aortic valve disease, albeit only associations exist about the role of increased circulating “EPC” levels in the preservation of valve integrity [149]. In contrast to “EPC”, circulating cells which co-express osteocalcin and bone alkaline phosphatase, where identified to actively promote calcification hyperglycaemia and diabetes. Of note, those myeloid calcifying cells (MCC) were distinct from HSC/“EPC” according to their antigenic profile, including lack of CD34 expression [150]. However, the derivation of MCC from cultured PBMC, albeit under growth conditions distinct from EOC, suggests inclusion of MCC screening into the panel of characterization for EOC used for cell therapy. Lack of comparable characterization data currently precludes conclusions about the modulation of MCC in CVD and therapy strategies, such as statin treatment or exercise training [150, 151]. Resident vascular cells, such as SMC or pericytes might also differentiate into calcifying cells under certain milieu conditions [152]. Exercise-mediated maintenance or restoration of endothelial confluence - possibly via “EPC”-mediated mechanisms – might help to counteract this process [151].

Much attention has been paid to the role of diet in retaining endothelial integrity and function, including unsaturated fatty acids, carbohydrate content and flavonoids [153-155]. Flavonoids present in red wine or cocoa, such as resveratrol or epocatechin, increase NO generation and reduce oxidative stress [156-158]. In vitro, as well as in pre-clinical and clinical studies, red wine, but not ethanol, enhanced “EPC” survival and increased vascularisation of ischemic tissues, mostly ascribed to NO mediated mechanisms [156, 158160]. In human CAD patients, intake of flavanol-rich cocoa increased NO production and the abundance of circulating “EPC” and improved endothelial function [161].

In summary, EOCs as well as circulating and vascularresident S/PC may protect from atherosclerosis by preserving critical endothelial functions. Due to the chronic course of plaque development, the beneficial effect of single cell application might turn out to be limited. Furthermore, the availability of pro-atherosclerotic and calcifying elements within the heterogeneous populations of PBMC and BMC, bears the risk of advancing, rather than counteracting atherosclerosis. Therefore, modulating the systemic condition towards a reduction of inflammation and strengthening of antioxidative defence

and promoting reverse cholesterol transport via systemic treatments holds greater promise. Resident or circulating “EPCs” will be among the cells benefiting, with niches protected, trafficking improved and differentiation driven away from pro-inflammatory directions.

3.3. “EPCs” for Cardiac Repair

The preservation of functional myocardium after AMI requires a fast restoration of reperfusion, as well as supplication of contractile and structural elements i.e. cardiomyocytes (CM) and fibrocytes. Due to their ability to enhance angiogenesis in ischemic situations, EOC, as well as various other BM-derived (stem) cell populations are regarded as promising tools for post-MI therapy [9, 162-164].

While in individual clinical studies and meta-analyses a small, but significant benefit of cell therapy for left ventricular (LV) function has been suggested, not all trials were able to detect significant improvement in cardiac function [165171]. The differential outcome of those studies has been attributed to technical differences, especially isolation and transport/storage procedures, yielding cell populations with distinct composition and functional capacity, but also to the time point and mode of application, as well as patients characteristics [48, 67, 172].

For practical reasons, most clinical studies have employed total BMC, whose cellular composition is rather mixed, including e.g. MSC, HSC, and stromal components. In consequence, various cellular and molecular mechanisms

- in detail explored by using more defined cell populations in vitro or in animal models - might contribute to the observed salvage on myocardial function in patients receiving autologous BMC. “EPC” differentiation into functional CM is still debated and the main effect of BMCs in myocardial salvage after MI is likely held by promotion of angiogenesis [162, 173-184]. For MSCs contained within the BMC some cardiopoietic potential, as well as the capacity to affect conductance, have been suggested and appears to be promoted by a “cocktail” of cytokines [185, 186]. Few comparative clinical studies have investigated the relative potential of total BMC in comparison to more defined cell populations, with heterogeneous outcome. Similar efficiency was attested to EOC or CD34+CXCR4+ cells as compared to unselected BMC [164, 167, 187, 188]. To overcome low engraftment and in order to deliver a relevant number of efficient cells, high numbers of total BMC are usually transplanted. Thereby, higher absolute numbers of contaminating, potentially inflammatory cells might be coinjected, possibly impeding the healing effect and triggering adverse events [188, 189]. To further optimize the efficacy of cell engraftment after transplantation, various routes of cell delivery have been used, including intracoronary, direct intracardiac (surgical transepicardial or catheter-based transendocardial) or intravenous injection. The appropriate method likely depends on the clinical scenario (i.e. acute infarction vs. chronic ischemic disease) and on the cell types used. Some studies have suggested a higher cell retention rate after intramyocardial injection, which allows targetted delivery, aided by mapping systems [190]. However, cell retention is generally low: Even with direct intramyocardioal injection, only around 10% of the injected cells are retained within the first hour of transplantation, with a further decline over the next days [191, 192]. Several groups therefore currently focus on enhancing the retention of injected cells, including delivery of cells within matrices, improvement of the microenvironment (i.e. recapitulating “niche-like” conditions), and ex vivo cell modifications (e.g. induction of adhesion molecules, improvement of survival) [193-199].

Recent meta-analyses suggest that transplantation of total autologous BMCs may improve LVEF, but not left ventricular remodelling, and their potency depends on the donor/recipient characteristics, with active smoking, male gender, age and diabetes negatively influencing efficiency [200203]. As donor and recipient necessarily are the same person, it is difficult in clinical studies to discern the impact of the milieu, the cells are exposed to after implantation, from intrinsic dysfunctions of the cells. In vitro studies and ex vivo analysis of transplanted cells indicate that both are relevant. Transplanted cells and their surroundings mutually interact, and therefore improving transplanted cells' function ex vivo (Table 1) together with pharmacological and/or lifestylebased lowering of systemic inflammatory status (Table 2) might be necessary to increase efficiency of autologous cellbased therapy after myocardial infarction.

Myocardial remodelling after acute infarction is characterised by two distinct phases in short to mid-term, with fibrotic processes stretching on to longer time periods [33, 204]. The mainly inflammatory conditions within the first week, characterised by apoptosis/necrosis and phagocytosis, in opposition to regenerative/angiogenic processes which occur during the following weeks, might serve to explain why cell application may be more effective in improving LVEF at ..6 days as compared to ..3 days after hospitalization [48]. After establishment of cardiomyopathy, injection of autologous BMC was still able to reduce infarct size and improve ventricular function [205]. Similar to other cell types, EOC or progenitors become dysfunctional and apoptotic when exposed to high levels of TNF-.. or IL-1.., conditions occurring immediately after aMI [206, 207]. Transplanted cells further suffer from the still ischemic conditions at the target site [208]. In contrast, inflammatory cytokines decrease in the second and third week after infarction, supporting “EPC” persistence. In the blood of patients successfully undergoing cardiac rehabilitation, increased availability of CD34+KDR+ PC was described, which correlated with systemic VEGF, IL-8 and IL-10 levels [209]. Therefore, conditions early after infarction appear to be detrimental to cell survival and function, explaining better effects with later time points of transplantation [48]. Vice versa, dysfunctional PC/EOC may also supply inflammatory cytokines, with adverse effects on myocardial healing and possible arrhythmogenic potential through paracrine stimulation of adjacent CM [185, 210]. Therefore, combination of cell application with statin therapy might yield synergistic effects, due to the systemic anti-inflammatory effects of statins, as well as specific effects on “EPC” survival and function e.g. differentiation [81, 206, 211]. Physical exercise, through multiple cellular and molecular mechanisms, including antioxidant and anti-inflammatory effects, is able to improve measures of cardiac function and geometry in heart failure patients and might also increase the success of stem cell transplantation [212]. In addition to their paracrine effects, electrophysiological alterations after cell transplantation have been debated and this depends on the cell type used [213]. Mechanistically, a lack of electrical coupling and distinct conductance characteristics between injected cells and the surrounding myocardium has been suggested to underlie arrhythmias, as suggested by various preclinical models i.e. co-culture and animal studies [214, 215]. Both, the lineage identity and stage of differentiation of the transplanted cell [216-218], as well as the position it is deposited at (surrounding myocardium viable or dead? [219]) affect electrical coupling to the host myocardium. For cardiopoietic cell types, the differentiation into various distinct CM lineages with individual action potential furthermore accounts for the occurrence of arrythmias [220]. However, the electrophysiological implications of cell therapy, as well as underlying cell-type specific

mechanisms are not entirely clear today and several recent clinical studies observed either no signs for pro-arrhythmic effects or even report anti-arrhythmic effects of stem-cell based approaches [221-224].

Due to their paracrine action, EOCs or other progenitor cell types, may affect all cell types in the vicinity, EC as well as CM, local SC niches or fibrocytes. In this context, their effect seems to be limited by the distance to the targeted CMs as well as by the length of their persistence [37, 225, 226]. Hence, higher numbers of recruited/persistent cells and uniform distribution along the target zone are aimed for in the clinical situation [227-231]. To ensure uniform high therapeutic quality of clinical stem cell therapy, optimized and standardized cell preparation, transport, and storage protocols need to be established. First experience on logistics (including equipment and training of personnel) and quality issues are available now from multicentre trials [232]. Finally, relevant outcome parameters defining the success of stem cell therapy and - potentially - its superiority vs. other therapies need to be agreed upon in order to compare individual approaches [233].

Cell therapy after MI, even after optimization of cell function, engraftment and survival, might still meet its limit through the impotency of this therapy approach to supply sufficiently high numbers of structural and contractile elements. Surgical approaches aiming specifically at improving structural stability and contractile force -especially for the long-term prevention of heart failure -include ex vivo engineered patches of various biomaterials, optionally seeded with cells and/or growth factors [234, 235]. Although still facing many difficulties on its way to clinical applications, this approach might prove more efficient in patients with advanced heart failure and a severe loss of contractile cardiomyocytes [236, 237]. The demands upon the material serving as matrix are high: it should not elicit host inflammatory response, feature conductance and mechanical properties similar to the host myocardium, and be stable over long time periods under repetitive physical strain [238]. Therefore, scaffold-free cell patches might constitute an alternative approach [239, 240]. Yet, long-term engraftment and stability issues, as well as connection to blood supply may also complicate the development of scaffold-free cardiac patches, so far limiting patch thickness/size [241]. Another obstacle to overcome for both approaches consists in ensuring proper electrical coupling of the transplanted patches to avoid arrhythmia, similar as discussed above for the transplantation of single-cell suspensions [242]. Here, the maturity of the cell type, determining distinct calcium current profiles, is critical [216-218]. While immature cells over time integrate with their neighbour cells, the risk of tumour formation or calcification is increased when embryonic undifferentiated SCs are used [53, 243]. In contrast, electrical coupling remains problematic with pre-differentiated cardiomyocytes. It remains to be seen whether the ex vivo de-differentiated inducible pluripotent stem (iPS) cells offer a better source for the generation of autologous tissue patches [244-246]. Yet, reprogramming of cells derived from CVD patients might be limited by epigenetic changes and DNA damage due to their levels of oxidative stress. Recent pioneering studies have established that epigenetic modifications are carried over to the iPS cell and that reprogramming may introduce aberrant DNA methylation, potentially affecting functionality and/or genetic stability of the reprogrammed cells [247, 248].

4. SUMMARY AND CONCLUSION

Current clinical data support a small but significant benefit of autologous cell transplantation with respect to cardiac function in the setting of acute myocardial infarction. However, the study outcome was divergent with regard to the extent of benefit, and in some cases did not support superiority of stem cell transplantation. Critical points to optimize in future trials are the functionality of the transplanted cells, milieu conditions within the recipient tissue, and rate of cell engraftment. In the setting of post-MI treatment, the main effect of HSC/“EPC” among the BMCs seems to consist in accelerating angiogenesis, thus enhancing perfusion and providing better survival conditions to remaining cardiac cells. Myeloid cells among the “EPC”/BMC might also modulate milieu conditions, such as the balance between inflammatory and repair-promoting cytokines, or nitric oxide and oxygen radical levels.

Preservation of S/PC niches within the bone marrow and possibly also the modulation of their differentiation, function and survival can be targeted by drugs, such as statins, as well as lifestyle adaptation. In patients with established CVD e.g. statin treatment or physical exercise, was able to increase “EPC” availability within the blood and their cellular functions. Furthermore, CD34+ cell-capturing stents combined with drug-eluting design are currently entering clinical trials, and may represent a novel approach to accelerate stent healing in the coronary artery.

The safety of different cell-based treatment approaches needs to be closely monitored with respect to the occurrence of adverse events, such as arrythmias or cell transplantationinduced promotion of neointima formation.

ACKNOWLEDGEMENT

The work is supported by a grant from the Uniscientia Stiftung, Liechtenstein. N.K. and U.L. are supported by the Swiss National Science Foundation.

Figures

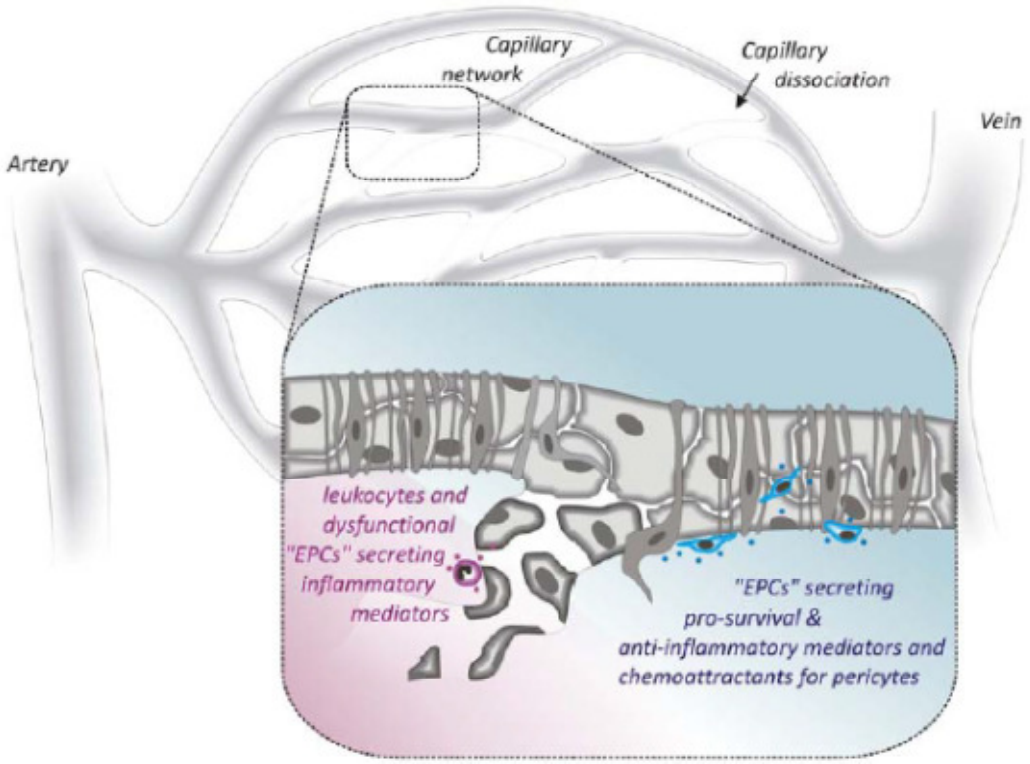

Figure 1. Potential effects of “endothelial progenitor cells (EPC)” on microvascular disease: In conditions with high oxidative stress e.g. in diabetes or aging, microvascular cells (i.e. EC, pericytes) die resulting in dissociation of microvessels. Extravasating leukocytes and dysfunctional “EPC”/early outgrowth cells (EOC) might aggravate this process by the secretion of inflammatory mediators, such as cytokines or reactive oxygen species (purple). Healthy “EPC”/EOC might counteract the loss of endothelial cells in several ways, including secretion of pro-survival factors such as nitric oxide or interleukin 10 and by supporting vessel stability by attracting pericytes (blue).

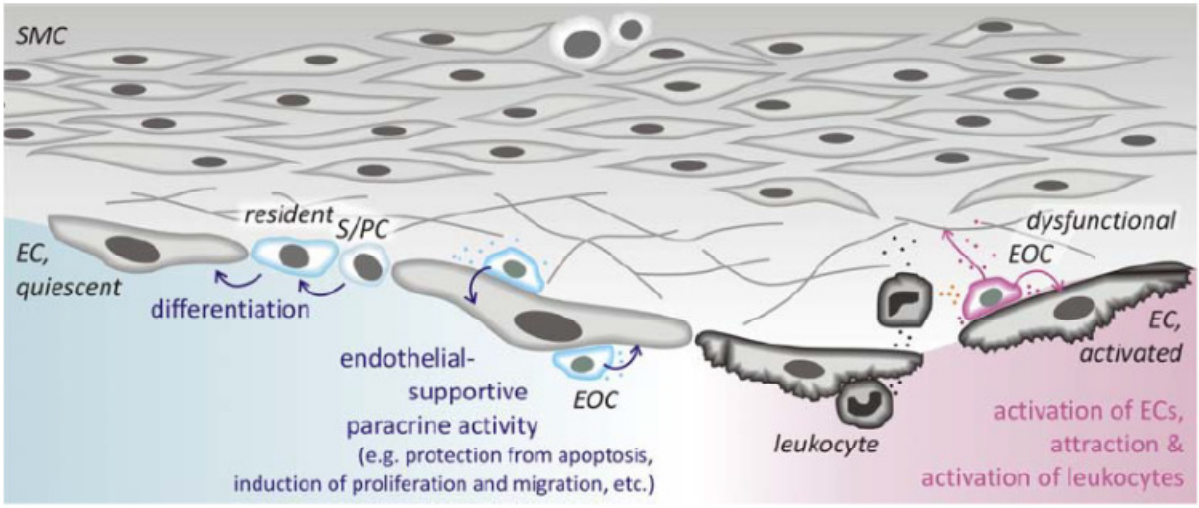

Figure 2: Potential effects of "endothelial progenitor cells (EPC)" in macrovascualr disease: Functional "EPC"/early outgrowth cells (EOC) from healthy individuals (blue) are involved in maintaining endothelial integrity e.g. by paracrinally inducing nitric oxide generation or protecting endothelial cells (EC) from apoptosis, while dysfunctional EOC, as e.g. in diabetic patients, might also promote inflammatory responses via secretion of inflammatory mediators and chemoattractants (purple). SMC: smooth muscle cells.

Tables

Targeted Mechanism	Targeted Pathway/Molecule	Outcome
"EPC" recruitment to target tissue (adhesion/invasion)	Growth factors (GF) (*ex vivo* overexpression in EOC or *ex vivo* treatment with GF protein, e.g. insulin-like GF 1 (IGF-1) or stromal cell-derived factor 1 (SDF-1))	IFG-1: reduced cardiomyocyte (CM) apoptosis, increased CM proliferation [73] improved migratory capacity, incorporation into forming vascular networks [249] SDF-1: enhanced recruitment of "EPC" [250] enhanced migration [251], improved engraftment/persistence [234] vascular endothelial GF (VEGF): enhanced recruitment to ischemic tissue in mouse model [72]
	PI3K/Akt/nitric oxide (NO) (*ex vivo* statin treatment, *ex vivo* overexpression of endothelial NO synthase (eNOS) in EOC)	*Statins:* enhanced migration [82] reverted loss of EOC adhesive and migratory capacity induced by oxidated low-density lipoproteins (oxLDL) [252] eNOS: increased migration and incorporation into network structures in matrigel [74]
	Hypoxia-induced signalling (*ex vivo* hypoxia-inducible factor 1a (HIF-1a) overexpression in EOC)	Improved "EPC" homing [253]
	Kallikrein-Kinin-System (*ex vivo* combined overexpression of human tissue kallikrein and kinin B2 receptor in EOC from type 2 diabetic patients)	Increased invasion of extracellular matrix gel [88]
	Prostanoids (*ex vivo* treatment of EOC with prostaglandin analogues)	Improved migration [254]
	Cyclooxygenase (COX) (*ex vivo* inhibition by aspirin, ibuprofen, parecoxib)	COX-1 inhibition either dose-dependently promotes "EPC" migration and adhesion [133] or decreased migratory and adhesive capacity of "EPC" [255]
	Polysaccharides (*ex vivo* exposure to fucoidan)	Enhanced expression of CXC receptor 4 (CXCR4) on CD34+ cells [256], enhanced migration and network formation [257]
	Growth hormones (*ex vivo* erythropoietin (Epo) treatment)	Enhanced adhesion to fibronectin [258]
"EPC" survival/ proliferation	angiogenic GF (*ex vivo* overexpression in EOC or *ex vivo* treatment with GF protein)	IFG-1: reduced cardiomyocyte (CM) apoptosis, increased CM proliferation, improved cardiac function [73] attenuated cellular senescence [249] VEGF: prevented "EPC" apoptosis [259] SDF-1: attenuates EOC apoptosis *ex vivo* [260] reduces senescence, increases proliferation [261] platelet-derived GF (PDGF): enhances *ex vivo* expansion of CD34+ progenitor cells (PC) [71]
	PI3K/Akt/NO (*ex vivo* overexpression in EOC)	statins: enhanced survival/cell cycling [80] prevented EOC apoptosis induced by oxLDL [252] eNOS: increased ^{3}H-thymidine incorporation (i.e. proliferation/DNA repair) [74]
	Growth hormones (*ex vivo* estrogen or Epo treatment)	Estrogen: reduced cellular senescence [262] Epo: enhanced proliferation
	Glycogen synthase kinase 3b (GSK-3b (*ex vivo* GSK-3b inhibitor treatment of EOC)	Reduced apoptosis [263]
	Prostanoids (*ex vivo* treatment of EOC with prostaglandin analogues)	alprostadil reduced apoptosis, improved proliferation [254]
	Anti-oxidant defense (*ex vivo* melantonin exposure)	Melatonin induces an overexpression of catalase and superoxide dismutase-1 and improves survival (264]
	Polysaccharides (*ex vivo* exposure to fucoidan)	Enhanced cell proliferation and migration [257]

Targeted Mechanism	Targeted Pathway/Molecule	Outcome
"EPC" differentiation/function	PI3K/Akt/NO (*ex vivo* statin treatment, *ex vivo* eNOS overexpression in EOC, *ex vivo* eNOS activation by AVE9488)	statins: anti-inflammatory polarization [81] reverted loss of EOC adhesive and migratory capacity induced by oxLDL [252] eNOS: reduction of neointimal hyperplasia [107], enhanced pro-angiogenic activity in mouse model [265]
	Growth factors (*ex vivo* overexpression in EOC or *ex vivo* treatment with GF protein)	IFG-1: improved cardiac function [73] SDF-1: better angiogenesis through enhanced recruitment of "EPC" [250] improved angiogenesis induction and preservation of cardiac function [234]
	Hypoxia-induced signalling (*ex vivo* HIF-1a overexpression in EOC)	Improved "EPC" homing, enhanced angiogenesis, reduced limb/toe necrosis [253]
	GSK-3b (*ex vivo* GSK-3b inhibitor treatment of EOC)	Attenuated neointima formation and enhanced re-endothelialization after arterial injury [263]
	Cyclooxygenase (*ex vivo* inhibition)	COX-1 inhibition decreased *in vitro* pro-vasculogenic capacity of "EPC" [255]
	Prostanoids (*ex vivo* treatment of EOC with prostaglandin analogues)	alprostadil improved revascularisation [254]
	anti-oxidant defence (*Ex vivo* manganese superoxide dismutase (Mn-SOD) overexpression, *ex vivo* melantonin exposure)	Mn-SOD: wound capillarization and closure improved [75], induction of angiogenesis and paracrine action improved [266] melatonin: enhanced network formation of ECs in matrigel in a paracrine manner [264]
	Polysaccharides (*ex vivo* exposure to fucoidan)	Enhanced cell proliferation, migration and network formation [257]
	Reverse cholesterol transport (*ex vivo* overexpression of Apolipoporotein A1 in EOC)	Reduction in atherosclerotic lesion area [103]

Table 1: Stategies for Ex Vivo Improvement of Autologous "Endothelial Progenitor Cells" (EPC) for Transplantation. Pre-Clinical And Clinical Results

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